Defining the p53 DNA-binding domain/Bcl-x_L-binding interface using NMR

Andrew M. Petros*, Angelo Gunasekera, Nan Xu, Edward T. Olejniczak, Stephen W. Fesik

Global Pharmaceutical Research and Development, Abbott Laboratories, 100 Abbott Park Rd., R46Y, AP10, Abbott Park, IL 60064, USA

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Abstract p53 exerts its tumor suppressor activity through both transcription-dependent and transcription-independent processes. Although the transcription-dependent activity of p53 has been extensively studied, the mechanism for transcription-independent p53-mediated tumor suppression is less well known. Recently, it was reported that p53 can directly induce mitochondrial permeabilization and promote apoptosis. This occurs through complexation of the DNA-binding region of p53 with the anti-apoptotic proteins Bcl-x_L and Bcl-2 (Mihara, M. et al. (2003) Mol. Cell 11, 577–590). Using nuclear magnetic resonance (NMR) spectroscopy we show that the interaction surface on p53 involves the same region that is used by the protein to contact DNA. The p53-binding site on Bcl-x_L consists of the carboxy-terminus of the first α-helix, the loop between α3 and α4, and the loop between α5 and α6 of Bcl-x_L. Furthermore, the interaction of p53 with Bcl-x_L is blocked by the binding of a 25-residue peptide derived from the BH3 region of the pro-apoptotic protein referred to as Bad.

Key words: Apoptosis; p53; Bcl-x_L; NMR

1. Introduction

The tumor suppressor protein p53 can trigger growth arrest or apoptosis in response to oncogenic stress such as DNA damage or hypoxia [1]. One way in which it acts is as a transcription factor, to either activate or suppress certain genes. However, several studies have suggested that the ability of p53 to induce apoptosis may not be solely dependent on its transcriptional role. For instance, several p53 mutants have been identified that are unable to regulate gene expression yet still retain the ability to induce apoptosis [2]. Furthermore, it has been shown that p53 can localize to the mitochondria and interact with anti-apoptotic Bcl-2 family members [2]. The Bcl-2 family of proteins, which are important regulators of apoptosis, modulate mitochondrial membrane integrity, at least in part, through heterodimerization of pro- and anti-apoptotic members. Mutants of p53 such as P72R, which localize to the mitochondrial membrane more than the wild-type protein, have an enhanced apoptotic potential [3]. The exact mechanism for p53-mediated apoptosis is still not clear, however, an interaction between p53 and Bcl-x_L or Bcl-2 could free pro-apoptotic Bax or Bak and thus induce changes in the mitochondrial membrane leading to the activation of the caspase cascade resulting in cell death.

We have investigated the protein–protein interaction between the DNA-binding domain of p53 and Bcl-x_L using nuclear magnetic resonance (NMR) spectroscopy. Complexation results in residue-specific spectral changes which define the points of contact between the two proteins. In addition, we have investigated the effect on complex formation of BH3 peptide binding to Bcl-x_L. These results are compared to a recently proposed model for the interaction surface of these two proteins and to known mutations of p53.

2. Materials and methods

2.1. Sample preparation

Bcl-x_L was expressed in *Escherichia coli* and purified as previously described [4]. A loop-deleted form of the protein was employed in which the long unstructured loop connecting α1 to α2 was truncated [4]. The anti-apoptotic potential of this protein is equivalent to the wild-type [4]. A uniformly ^{15}N,^{13}C-labelled sample was prepared with a minimal medium containing $^{15}NH_4Cl$ as the sole nitrogen source and [U-^{13}C]glucose as the sole carbon source.

The DNA-binding domain of p53 (residues 94–312) was polymerase chain reaction (PCR) amplified from a human kidney cDNA library (Clontech) using DNA primers (5′-ACGACGACGTCTCCCATGTCATCTTCTGTCCCTTCCCAG-3′ and 5′-CGAGAGACGTCTCCTCGAGGGTGTTGTTGGGCAGTGCTCGCTTAG-3′). The PCR product was restriction digested with *Bsm*BI (New England Biolabs), ligated into a modified pET15b vector (Novagen, WI, USA) with an amino-terminal His tag, and expressed in *E. coli* BL21/DE3 (Novagen). The protein was purified on an S-protein ion-exchange column (Bio-Rad) followed by affinity chromatography purification on a nickel-IDA column (Invitrogen). The amino-terminal His tag was left intact. A uniformly ^{15}N-labelled sample was prepared with minimal media containing $^{15}NH_4Cl$ as the sole nitrogen source. NMR samples typically contained 0.1–0.5 mM protein in phosphate-buffered saline with 10% ^2H_2O added.

2.2. NMR spectroscopy

NMR spectra were acquired at 298 K on either a Bruker DRX500 or DRX600 spectrometer, equipped with a cryoprobe accessory. Perturbations to ^{13}C-labelled Bcl-x_L upon binding unlabelled p53 DNA-binding domain were monitored using either a ^{13}C-heteronuclear single-quantum correlation (HSQC) or a ^{13}C-edited nuclear Overhauser effect spectroscopy (NOESY) spectrum recorded with a mixing time of 80 ms. Perturbations to the p53 DNA-binding domain upon binding unlabelled Bcl-x_L were monitored with an ^{15}N-HSQC, and the interpretation of the data was based on previously published assignments [5].

3. Results and discussion

In order to define the interaction surface for p53 on Bcl-x_L,

*Corresponding author. Fax: (1)-847-938 2478.
E-mail address: andrew.petros@abbott.com (A.M. Petros).

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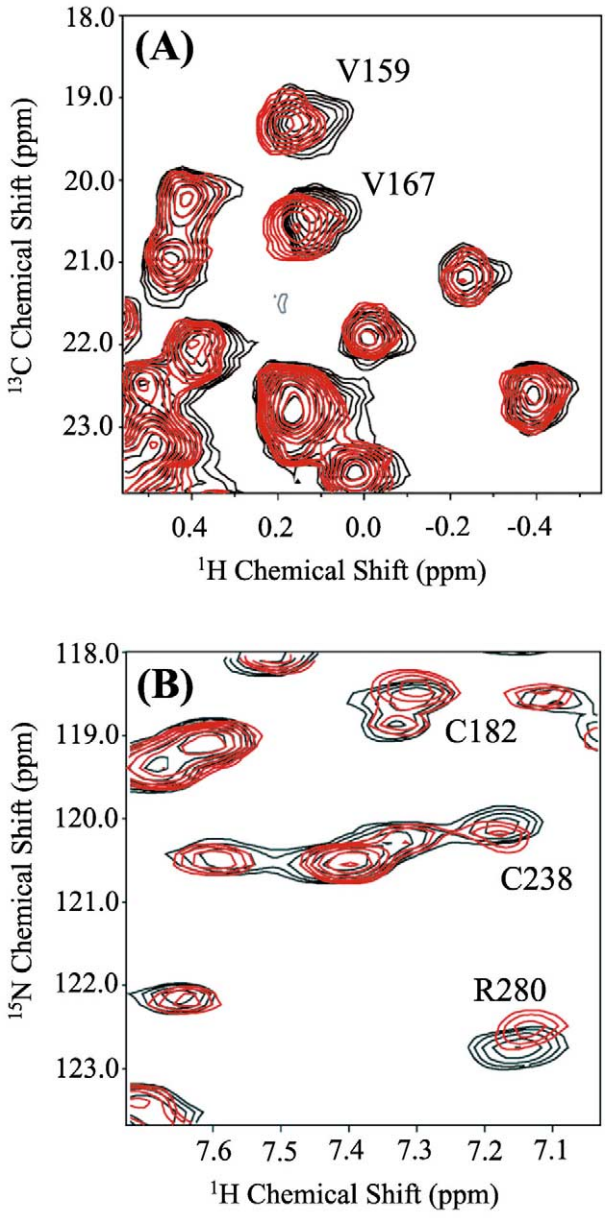

Fig. 1. A: Section of a ^{13}C-HSQC spectrum recorded on ^{13}C-labelled Bcl-x_L in the absence (black) and presence (red) of a slight molar excess of unlabelled p53 DNA-binding domain. Resonances of Bcl-x_L perturbed upon binding to the p53 DNA-binding domain are labelled. B: Section of a ^{15}N-HSQC spectrum recorded on ^{15}N-labelled p53 DNA-binding domain in the absence (black) and presence (red) of a slight molar excess of unlabelled Bcl-x_L. Resonances on p53 perturbed upon binding to Bcl-x_L are labelled.

we monitored spectral changes in a ^{13}C-HSQC spectrum and a ^{13}C-edited NOESY spectrum for a sample of uniformly ^{13}C-labelled Bcl-x_L complexed with unlabelled DNA-binding domain from p53. Overall, spectra recorded in the presence of p53 were very similar to those recorded on Bcl-x_L alone, suggesting that Bcl-x_L does not undergo a major conformation change upon binding. A representative section of the ^{13}C-HSQC spectrum for Bcl-x_L both alone and in the presence of the p53 DNA-binding domain is shown in Fig. 1A. As can be seen, Val-159 and Val-167 are specifically perturbed upon binding of p53 to Bcl-x_L. Additional perturbations observed in this spectrum and in a ^{13}C-edited NOESY spectrum of Bcl-x_L are summarized in Fig. 2A. These residues are localized to a small region of Bcl-x_L including the carboxy-terminus of α1, the loop between α3 and α4, and the loop between α5 and α6. They are not in the groove formed by the BH1, BH2, and BH3 regions of the protein that is the binding site for BH3 peptides, such as those derived from the anti-apoptotic proteins Bak and Bad, Fig. 2B. In Fig. 2C the surface of Bcl-x_L is shown color coded by atom type. The p53-binding surface has an acidic character, unlike the hydrophobic, BH3 peptide-binding groove.

The binding interface for Bcl-x_L on p53 was defined by adding unlabelled Bcl-x_L to ^{15}N-labelled p53 DNA-binding domain and monitoring the subsequent changes observed in an ^{15}N-HSQC spectrum. Since assignments for the backbone amide resonances of the p53 DNA-binding domain have been reported [5] while those for side chain atoms have not, this ^{15}N-based approach was necessary for p53. As with Bcl-x_L, perturbations to the HSQC spectrum were very specific, as shown in Fig. 1B, suggesting that the p53 DNA-binding domain does not undergo a major conformational change. The two proteins are in overall fast exchange on the NMR timescale as only a single signal is observed for those resonances of the p53 DNA-binding domain perturbed upon binding to Bcl-x_L and for those of Bcl-x_L perturbed upon binding to p53. This suggests that these proteins associate with a K_d greater than 1 uM. Fig. 3A shows the residues of the p53 DNA-binding domain that are significantly affected by addition of Bcl-x_L. The residues perturbed are localized to the same region that is used by p53 to contact DNA, Fig. 3B. In particular, Arg-280, which is perturbed upon binding to Bcl-x_L, interacts directly with the major groove of DNA. A molecular surface for the p53 DNA-binding domain is shown in Fig. 3C. The basic character of the interaction site is consistent with its role in binding DNA and would complement the acidic character of the Bcl-x_L surface shown in Fig. 2C. Several naturally occurring mutations to the p53 DNA-binding domain have been identified which abolish both the transcription-dependent and transcription-independent actions of p53 [2]. These mutations include R175H, L194F, R273H, and R280K, all of which lie within or are proximal to the Bcl-x_L interface as defined by the NMR data.

Our results are also consistent with a recently proposed model of Bcl-x_L binding to the p53 DNA-binding domain. This model was generated using the Global Range Molecular Matching Program [6]. In this model a loop from the p53 DNA-binding domain comprised of residues 239–248 interacts with a groove on Bcl-x_L formed by α1 and part of α2.

While the residues of the peptide-binding groove of Bcl-x_L were not perturbed upon complexation with p53, part of the loop between α3 and α4, which is proximal to this groove, is perturbed by addition of p53, Fig. 2B. Previous studies showed that α3 moves upon binding of BH3 peptides [4,7]. To test whether p53 can bind to Bcl-x_L in the presence of a BH3 peptide, we added the p53 DNA-binding domain to a preformed complex of ^{13}C-labelled Bcl-x_L with a 25-residue BH3 peptide from the pro-apoptotic Bad protein. This peptide binds to Bcl-x_L with a dissociation constant of 0.6 nM [7]. Upon subsequent addition of p53 no spectral changes were observed in the Bcl-x_L ^{13}C-HSQC spectrum, suggesting that the p53 DNA-binding domain does not bind in the presence of the Bad, BH3 peptide. These data suggest that BH3 peptide binding to Bcl-x_L can influence the binding of p53. Peptides

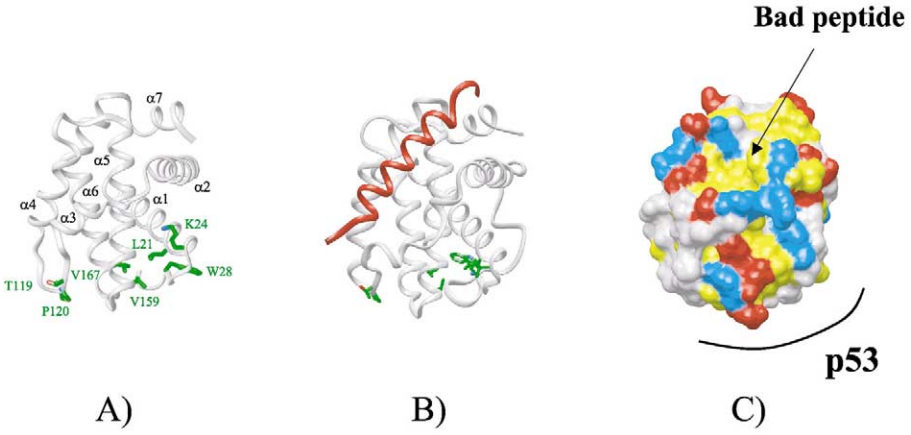

Fig. 2. A: Ribbon representation of Bcl-x_L. Residues perturbed upon interaction with the p53 DNA-binding domain are shown in green. B: Ribbon representation of Bcl-x_L complexed with a 25-residue peptide from the BH3 region of the pro-apoptotic Bad protein (red). The binding groove of Bcl-x_L is composed of α2, α3, α4, and the loop connecting α4 to α5. C: Solvent-accessible surface of Bcl-x_L showing p53 interface and hydrophobic groove. Leucine, valine, isoleucine, phenylalanine, tyrosine, tryptophan, methionine, and alanine are colored yellow; arginine, lysine, and histidine are colored blue; aspartic acid and glutamic acid are colored red, while all other residues are colored gray. As can be seen, the p53 interface has an overall acidic character.

from Bak and Bax which bind much weaker to Bcl-x_L, 480 and 13000 nM respectively [8], may not be able to block P53 binding. Indeed, in vivo immunoprecipitation studies on the binding of p53 to Bcl-x_L [2] suggested that overexpression of the pro-apoptotic Bax protein does not block complex formation of Bcl-x_L with p53. Additional studies need to be done to investigate the effect of the different BH3 proteins on the Bcl-x_L/p53 interaction.

4. Conclusions

Recent data have suggested a direct interaction between Bcl-x_L and p53 at the mitochondrial membrane. Using NMR-based chemical shift mapping, we have investigated the structural basis of this interaction and have experimentally defined the relevant interaction surfaces on both proteins. Furthermore, we have used peptides that mimic the interaction between pro-apoptotic proteins and Bcl-x_L to test their effect on the Bcl-x_L/p53 interaction. These new data provide information at the molecular level on this additional mechanism for the regulation of apoptosis that is initiated by p53.

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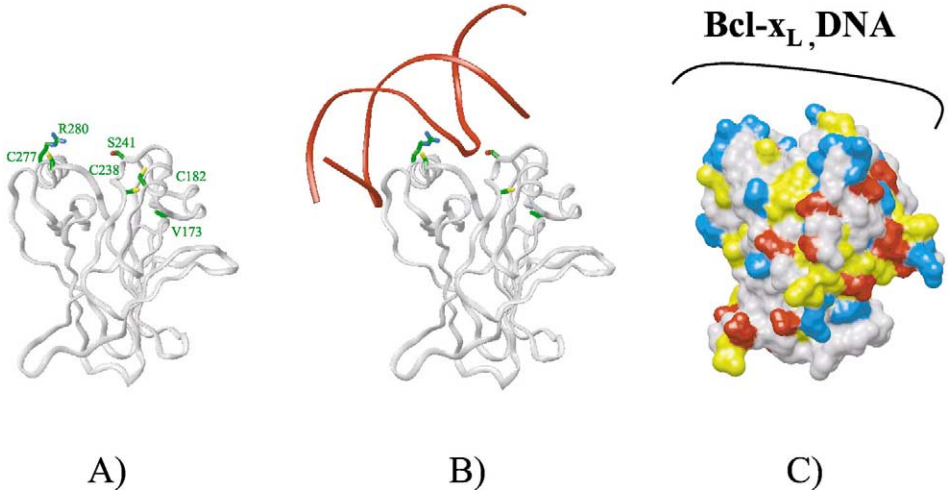

Fig. 3. A: Ribbon representation of p53 DNA-binding domain. Residues whose amide resonances are perturbed upon interaction with Bcl-x_L are shown in green. B: Ribbon representation of p53 DNA-binding domain complexed with DNA (red). C: Solvent-accessible surface of the p53 DNA-binding domain. Leucine, valine, isoleucine, phenylalanine, tyrosine, tryptophan, methionine, and alanine are colored yellow; arginine, lysine, and histidine are colored blue; aspartic acid and glutamic acid are colored red, while all other residues are colored gray. As can be seen, the Bcl-x_L interface has an overall basic character and in fact, this is the same general region of p53 which associates with DNA.

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